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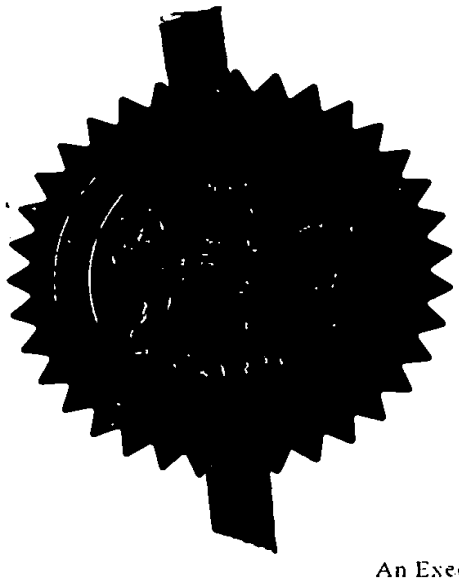

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Patents ADP number *(if you know it)* 73841340001

If the applicant is a corporate body, give the country/state of its incorporation — UK

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5. Name of your agent *(if you have one)*

Murgitroyd & Company

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"PEPTIDE"

The present invention relates to release of insulin and control of blood glucose concentration. More particularly the invention relates to the use of peptides to stimulate release of insulin, lowering of blood glucose and pharmaceutical preparations for treatment of type 2 diabetes.

Gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1(7-36)amide (truncated GLP-1; tGLP-1) are two important insulin-releasing hormones secreted from endocrine cells in the intestinal tract in response to feeding [1,2]. Together with autonomic nerves they play a vital supporting role to the pancreatic islets in the control of blood glucose homeostasis and nutrient metabolism [1,3].

Dipeptidyl peptidase IV (DPP IV; EC 3.4.14.5) has been identified as a key enzyme responsible for inactivation of GIP and tGLP-1 in serum [4,5]. DPP IV is completely inhibited in serum by the addition of diprotin A(DPA, 0.1 mmol/l) [4]. This occurs through the rapid removal of the N-terminal dipeptides Tyr^1- Ala^2 and His^7-Ala^8 giving rise to the main metabolites GIP(3-42) and GLP-

1(9-36)amide, respectively. These truncated peptides are reported to lack biological activity or to even serve as antagonists at GIP or tGLP-1 receptors [6-9]. The resulting biological half-lives of these incretin hormones *in vivo* are therefore very short, estimated to be no longer than 5 min [5, 10-12].

In situations of normal glucose regulation and pancreatic B-cell sensitivity, this short duration of action is advantageous in facilitating momentary adjustments to homeostatic control. However, the current goal of a possible therapeutic role of incretin hormones, particularly tGLP-1 in NIDDM therapy is frustrated by a number of factors in addition to finding a convenient route of administration [13]. Most notable of these are rapid peptide degradation and rapid absorption (peak concentrations reached 20 min) and the resulting need for both high dosage and precise timing with meals [13-15]. Recent therapeutic strategies have focused on precipitated preparations to delay peptide absorption [16] and inhibition of GLP-1 degradation using specific inhibitors of DPP IV [17-19]. A possible therapeutic role is also suggested by the observation that a specific inhibitor of DPP IV, isoleucine thiazolidide, lowered blood glucose and enhanced insulin secretion in glucose-treated diabetic obese Zucker rats presumably by protecting against catabolism of the incretin hormones tGLP-1 and GIP [18].

Numerous studies have indicated that tGLP-1 infusion restores pancreatic B-cell sensitivity, insulin secretory oscillations and improved glycemic control in various groups of patients with IGT or NIDDM [13,15,20-22]. Longer term studies also show significant benefits of tGLP-1 injections in NIDDM and possibly

IDDM therapy [20,23,24], providing a major incentive to develop an orally effective or long-acting tGLP-1 analogue [13]. Several attempts have been made to produce structurally modified analogues of tGLP-1 which are resistant to DPP IV degradation [25-27]. A significant extension of serum half-life is observed with His^7- glucitol tGLP-1 and tGLP-1 analogues substituted at position 8 with Gly, Aib, Ser or Thr [25-27]. However, these structural modifications seem to impair receptor binding and insulinotrophic activity thereby compromising part of the benefits of protection from proteolytic degradation [25-28]. In recent studies using His^7-glucitol tGLP-1, resistance to DPP IV and serum degradation was accompanied by severe loss of insulin-releasing activity [26,28].

GIP shares not only the same degradation pathway as tGLP 1 but many similar physiological actions, including stimulation of insulin and somatostatin secretion, and the enhancement of glucose disposal [1]. These actions are viewed as key aspects in the antihyperglycemic properties of tGLP-1 [13], and there is therefore good expectation that GIP may have similar potential as NIDDM therapy. Indeed, compensation by GIP is held to explain the modest disturbances of glucose homeostasis observed in tGLP-1 knockout mice [29]. Apart from early studies [30], the anti-diabetic potential of GIP has not been explored and tGLP-1 may seem more attractive since it is viewed by some as a more potent insulin secretagogue when infused at "so called" physiological concentrations estimated by RIA [31].

It has been shown that N-terminal glycation of GIP markedly enhances the insulin releasing effect of the peptide on clonal B-cells [32].

The present invention aims to provide effective analogues of GIP. It is one aim of the invention to provide a pharmaceutical for treatment of Type 2 diabetes.

According to the present invention there is provided effective analogues of the biologically active GIP(1-42) which have improved characteristics for treatment of Type 2 diabetes wherein the analogues have amino acid substitutions at positions 1-3 and or contain various other amino acid substitutions in the basic peptide. The structures of human and porcine GIP(1-42) are shown below. The porcine peptide differs by just two amino acid substitutions at positions 18 and 34.

Fig. 1. Primary structure of human gastric inhibitory polypeptide (GIP)

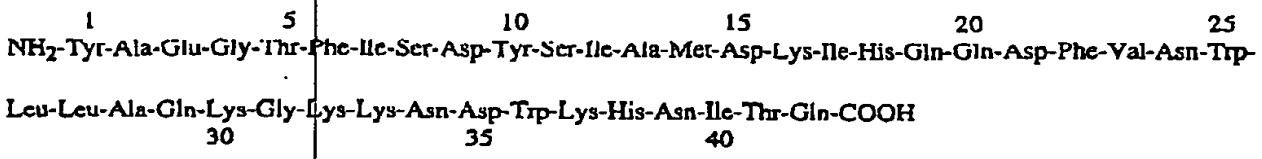
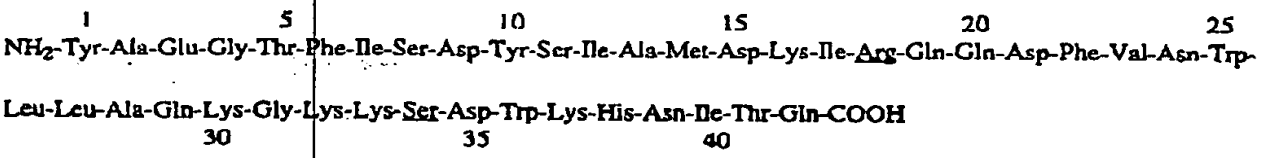


Fig. 2. Primary structure of porcine gastric inhibitory polypeptide (GIP)



The analogues of GIP(1-42) may have an enhanced capacity to stimulate insulin secretion, enhance glucose disposal, delay glucose absorption or may exhibit enhanced stability in plasma as compared to native GIP.

Any of these properties will enhance the potency of the analogue as a therapeutic agent.

Analogues having D-amino acid substitutions in the 1, 2 and 3 positions and/or N-glycated, N-alkylated, N-acetylated or N-acylated amino acids in the 1 position are resistant to degradation *in vivo*.

Various amino acid substitutions at second and third amino terminal residues are included, such as GIP(1-42)Gly2, GIP(1-42)Ser2, GIP(1-42)Abu2, GIP(1-42)Aib, GIP(1-42)D-Ala2, GIP(1-42)Sar2, and GIP(1-42)Pro3.

Amino-terminally modified GIP analogues include N-glycated GIP(1-42), N-alkylated GIP(1-42), N-acetylated GIP(1-42), N-acetyl-GIP(1-42) and N-isopropyl GIP(1-42).

Other stabilised analogues include those with a peptide isostere bond between amino terminal residues at position 2 and 3. These analogues may be resistant to the plasma enzyme dipeptidyl-peptidase IV (DPP IV) which is largely responsible for inactivation of GIP by removal of the amino-terminal dipeptide Tyr1-Ala2.

In particular embodiments, the invention provides a peptide which is more potent than human or porcine GIP in moderating blood glucose excursions, said peptide consisting of GIP(1-42) or N-terminal fragments of GIP(1-42) consisting of up to 30 amino acid residues from the N-terminus (i.e. GIP(1-30)) with one or more modifications selected from the group consisting of:

(a) substitution of Ala2 by Gly
(b) substitution of Ala2 by Ser
(c) substitution of Ala2 by Abu
(d) substitution of Ala2 by Aib
(e) substitution of Ala2 by D-Ala
(f) substitution of Ala2 by Sar

(g) substitution of Glu3 by Pro
(h) modification of Tyr^1 by acetylation
(i) modification of Tyr^1 by acylation
(j) modification of Tyr^1 by alkylation
(k) modification of Tyr^1 by glycation
(l) conversion of Ala2-Glu3 bond to a psi [CH2NH] bond
(m) conversion of Ala2-Glu3 bond to a stable peptide isotere bond
(n) (n-isopropyl-H) 1GIP.

A preferred embodiment of the invention provides the use of Tyr^1-glucitol GIP in the preparation of a medicament for the treatment of diabetes.

The invention further provides improved pharmaceutical compositions including analogues of GIP with improved pharmacological properties.

Other possible analogues include certain commonly encountered amino acids, which are not encoded by the genetic code, for example, beta-alanine (beta-ala), or other omega-amino acids, such as 3-amino propionic, 4-amino butyric and so forth, alpha-aminoisobutyric acid (Aib), sarcosine (Sar), ornithine (Orn), citrulline (Cit), homoarginine (Har), t-butylalanine (t-BuA), t-butylglycine (t-BuG), N-methylisoleucine (N-MeIle), phenylglycine (Phg), and cyclohexylalanine (Cha), norleucine (Nle), cysteic acid (Cya) and methionine sulfoxide (MSO), substitution of the D form of a neutral or acidic amino acid or the D form of tyrosine for tyrosine.

According to the present invention there is also provided a pharmaceutical composition useful in the treatment of diabetes type II which comprises an effective amount of the peptide as described herein, in

admixture with a pharmaceutically acceptable excipient.

The invention will now be demonstrated with reference to the following non-limiting example and the accompanying figures wherein:

Figure 1 illustrates degradation of GIP and Tyr^1-glucitol GIP by DPP IV.

Figure 2 illustrates degradation of GIP and Tyr^1-glucitol GIP by human plasma.

Figure 3 illustrates electrospray ionization mass spectrometry of GIP, Tyr^1-glucitol GIP and the major degradation fragment GIP(3-42).

Figure 4 shows the effects of GIP and glycated GIP on plasma glucose homeostasis.

Figure 5 shows Effects of GIP on plasma insulin responses (A).

Example

The following example investigates preparation of Tyr^1-glycitol GIP together with evaluation of its antihyperglycemic and insulin-releasing properties in vivo. The results clearly demonstrate that this novel GIP analogue extracts a substantial resistance to aminopeptidase degradation and increased glucose lowering activity compared with the native GIP.

Research Design and Methods

Materials. Human GIP was purchased from the American Peptide Company (Sunnyvale, CA, USA). HPLC grade

acetonitrile was obtained from Rathburn (Walkersburn, Scotland). Sequencing grade trifluoroacetic acid (TFA) was obtained from Aldrich (Poole, Dorset, UK). All other chemicals purchased including dextran T-70, activated charcoal, sodium cyanoborohydride and bovine serum albumin fraction V were from Sigma (Poole, Dorset, UK). Diprotin A (DPA) was purchased from Calbiochem-Novabiochem (UK) Ltd. (Beeston, Nottingham, UK) and rat insulin standard for RIA was obtained form Novo Industria (Copenhagen, Denmark). Reversed-phase Sep-Pak cartridges (C-18) were purchased from Millipore-Waters (Milford, MA, USA). All water used in these experiments was purified using a Milli-Q, Water Purification System (Millipore Corporation, Milford, MA, USA).

Preparation of Tyr^1-glucitol GIP. Human GIP was incubated with glucose under reducing conditions in 10 mmol/l sodium phosphate buffer at pH 7.4 for 24 h. The reaction was stopped by addition of 0.5 mol/l acetic acid (30 µl) and the mixture applied to a Vydac (C-18) (4.6 x 250mm) analytical HPLC column (The Separations Group, Hesperia, CA, USA) and gradient elution conditions were established using aqueous/TFA and acetonitrile/TFA solvents. Fractions corresponding to the glycated peaks were pooled, taken to dryness under vacuum using an AES 1000 Speed-Vac concentrator (Life Sciences International, Runcorn, UK) and purified to homogeneity on a Supelcosil (C-8) (4.6 x 150mm) column (Supelco Inc., Poole, Dorset, UK).

Degradation of GIP and Tyr^1-glucitol GIP by DPP IV. HPLC-purified GIP or Tyr^1-glucitol GIP were incubated at 37°C with DPP-IV (5mU) for various time periods in a reaction mixture made up to 500 µl with 50 mmol/l triethanolamine-HCl, pH 7.8 (final peptide

concentration 1 μmol/l) [4]. Enzymatic reactions were terminated after 0, 2, 4 and 12 hours by addition of 5 μl of 10% (v/v) TFA/water. Samples were made up to a final volume of 1.0 ml with 0.12% (v/v) TFA and stored at -20°C prior to HPLC analysis.

Degradation of GIP and Tyr^1-glucitol GIP by human plasma. Pooled human plasma (20 μl) taken from six healthy fasted human subjects was incubated at 37°C with GIP or Tyr^1-glucitol GIP (10 μg) for 0 and 4 hours in a reaction mixture made up to 500 μl, containing 50 mmol/l triethanolamine/HCL buffer pH 7.8. Incubations for 4 hours were also performed in the presence of diprotin A (5 mU). The reactions were terminated by addition of 5 μl of TFA and the final volume adjusted to 1.0 ml using 0.1% v/v TFA/water. Samples were centrifuged (13,000g, 5 min) and the supernatant applied to a C-18 Sep-Pak cartridge (Millipore-Waters) which was previously primed and washed with 0.1% (v/v) TFA/water. After washing with 20 ml 0.12% TFA/water, bound material was released by elution with 2 ml of 80% (v/v) acetonitrile/water and concentrated using a Speed-Vac concentrator (Runcorn, UK). The volume was adjusted to 1.0ml with 0.12% (v/v) TFA/water prior to HPLC purification.

HPLC analysis of degraded GIP and Tyr^1-glucitol GIP. Samples were applied to a Vydac C-18 widepore column equilibriated with 0.12% (v/v) TFA/H_2O at a flow rate of 1.0 ml/min. Using 0.1% (v/v) TFA in 70% acetonitrile/H_2O, the concentration of acetonitrile in the eluting solvent was raised from 0% to 31.5% over 15 min, to 38.5% over 30 min and from 38.5% to 70% over 5 min, using linear gradients. The absorbance was monitored at 206 nm and peak areas evaluated using a model 2221 LKB integrator. Samples recovered manually

were concentrated using a Speed-Vac concentrator.

Electrospray ionization mass spectrometry (ESI-MS). Samples for ESI-MS analysis containing intact and degradation fragments of GIP (from DPP IV and plasma incubations) as well as Tyr^1-glucitol GIP, were further purified by HPLC. Peptides were dissolved (approximately 400 pmol) in 100 μl of water and applied to the LCQ benchtop mass spectrometer (Finnigan MAT, Hemel Hempstead, UK) equipped with a microbore C-18 HPLC column (150 x 2.0mm, Phenomenex, UK, Ltd, Macclesfield). Samples (30μl direct loop injection) were injected at a flow rate of 0.2ml/min, under isocratic conditions 35% (v/v) acetonitile/water. Mass spectra were obtained from the quadripole ion trap mass analyzer and recorded. Spectra were collected using full ion scan mode over the mass-to-charge (m/z) range 150-2000. The molecular masses of GIP and related structures were determined from ESI-MS profiles using prominent multiple charged ions and the following equation $M_r = iM_i - iM_h$ (where M_r = molecular mass; M_i = m/z ratio; i = number of charges; M_h = mass of a proton).

***In vivo* biological activity of GIP and Try^1-glucitol GIP.** Effects of GIP and Tyr^1-glucitol GIP on plasma glucose and insulin concentrations were examined using 10-12 week old male Wistar rats. The animals were housed individually in an air conditioned room and 22±2°C with a 12 hour light/12 hour dark cycle. Drinking water and a standard rodent maintenance diet (Trouw Nutrition, Belfast) were supplied *ad libitum*. Food was withdrawm for an 18 hour period prior to intraperitoneal injection of glucose alone (18mmol/kg body weight) or in combination with either GIP or Tyr^1-glucitol GIP (10 nmol/kg). Test solutions were

administered in a final volume of 8 ml/kg body weight. Blood samples were collected at 0, 15, 30 and 60 minutes from the cut tip of the tail of conscious rats into chilled fluoride/heparin microcentrifuge tubes (Sarstedt, Nümbrecht, Germany). Samples were centrifuged using a Beckman microcentrifuge for about 30 seconds at 13,000 g. Plasma samples were aliquoted and stored at -20°C prior to glucose and insulin determinations. All animal studies were done in accordance with the Animals (Scientific Procedures) Act 1986.

Analyses. Plasma glucose was assayed by an automated glucose oxidase procedure using a Beckman Glucose Analyzer II [33]. Plasma insulin was determined by dextran charcoal radioimmunoassay as described previously [34]. Incremental areas under plasma glucose and insulin curves (AUC) were calculated using a computer program (CAREA) employing the trapezoidal rule [35] with baseline subtraction. Results are expressed as mean ± SEM and values were compared using the Student's unpaired *t*-test. Groups of data were considered to be significantly different if $P<0.05$.

Results

Degradation of GIP and Tyr^1-glucitol GIP by DPP IV. Figure 1 illustrates the typical peak profiles obtained from the HPLC separation of the products obtained from the incubation of GIP (left panels) or Tyr^1-glucitol GIP (right panels) with DPP IV for 0, 2, 4 and 12 hours. The retention times of GIP and Tyr^1-glucitol GIP at t=0 were 21.93 minutes and 21.75 minutes respectively. Degradation of GIP was evident after 4 hours incubation (54% intact), and by 12 hours the majority (60% of intact GIP was converted to the single product with a

retention time of 21.61 minutes. Tyr^1-glucitol GIP remained almost completely intact throughout 2-12 hours incubation.

Degradation of GIP and Tyr^1-glucitol GIP by human plasma. Figure 2 shows a set of typical HPLC profiles of the products obtained from the incubation of GIP or Tyr^1-glucitol GIP with human plasma for 0 and 4 h. GIP (left panels) with a retention time of 22.06 min was readily metabolised by plasma within 4 hours incubation giving rise to the appearance of a major degradation peak with a retention time of 21.74 minutes. In contrast, the incubation of Tyr^1-glucitol GIP under similar conditions (right panels) did not result in the formation of any detectable degradation fragments during this time with only a single peak being observed with a retention time of 21.77 minutes. Addition of diprotin A, a specific inhibitor of DPP IV, to GIP during the 4 hours incubation completely inhibited degradation of the peptide by plasma.

Identification of GIP degradation fragments by ESI-MS. Figure 3 shows the monoisotopic molecular masses obtained for GIP, (panel A), Tyr^1-glucitol GIP (panel B) and the major plasma degradation fragment of GIP (panel C) using ESI-MS. The peptides analyzed were purified from plasma incubations as shown in Figure 2. The exact molecular mass (M_r) of the peptides were calculated using the equation $M_r = iM_l - iM_h$ as defined in Research Design and Methods section. After spectral averaging was performed, prominent multiple charges species $(M+3H)^{3+}$ and $(M+4H)^{4+}$ were detected from GIP at m/z 1661.6 and 1246.8, corresponding to intact M_r 4981.8 and 4983.2 Da, respectively (Fig. 3A). Similarly, for Tyr^1-glucitol GIP ($(M+4H)^{4+}$ and $(M+5H)^{5+}$) were detected at m/z 1287.7 and 1030.3, corresponding to intact

molecular masses of M^r 5146.8 and 5146.5 Da, respectively (Fig. 3B). The difference between the observed molecular masses of the quadruply charged GIP and the N-terminally modified GIP species (163.6 Da) indicated that the latter peptide contained a single glucitol adduct corresponding to Tyr^1-glucitol GIP. Figure 3C shows the prominent multiply charged species $(M+3H)^{3+}$ and $(M+4H)^{4+}$ detected from the major fragment of GIP at m/z 1583.8 and 1188.1, corresponding to intact M^r 4748.4 and 4748 Da, respectively. This corresponds with the theoretical mass of the N-terminally truncated form of the peptide GIP(3-42). This fragment was also the major degradation product of DPP IV incubations (data not shown).

Effects of GIP and Tyr^1-glucitol GIP on plasma glucose homeostasis. Figures 4 and 5 show the effects of intraperitoneal glucose alone (18mmol.kg) (control group), and glucose in combination with GIP or Tyr^1-glucitol GIP (10nmol/kg) on plasma glucose and insulin concentrations. Compared with the control group, plasma glucose concentrations and area under the curve (AUC) were significantly lower following administration of either GIP or Tyr^1- glucitol GIP (Figure 4A, B). Furthermore, individual valus at 15 and 30 minutes together with AUC were significantly lower following administration of Tyr^1-glucitol GIP as compared to GIP. Consistent with the established insulin-releasing properties of GIP, plasma insulin concentrations of both peptide-treated groups were significantly raised at 15 and 30 minutes compared with the values after administration of glucose alone (Figure 5A). The overall insulin responses, estimated as AUC were also signigicantly greater for the two peptide-treated groups (Figure 5B). Despite lower prevailing glucose concentrations than the GIP-treated group, plasma

insulin response, calculated as AUC, following Tyr^1-glucitol GIP was significantly greater than after GIP (Figure 5B). The significant elevation of plasma insulin at 30 minutes is of particular note, suggesting that the insulin-releasing action of Tyr^1-glucitol GIP is more protracted than GIP even in the face of a diminished glycemic stimulus (Figures 4A, 5A).

Discussion

The forty two amino acid GIP is an important incretin hormone released into the circulation from endocrine K-cells of the duodenum and jejunum following ingestion of food [36]. The high degree of structural conservation of GIP among species supports the view that this peptide plays and important role in metabolism [12]. Secretion of GIP is stimulateed directly by actively transported nutrients in the gut lumen without a notable input from autonomic nerves [12]. The most important stimulants of GIP release are simple sugars [37] and unsaturated long chain fatty acids [38], with amino acids exerting weaker effects [39]. As with tGLP-1, the insulin-releasing effect of GIP is strictly glucose-dependent [30,40]. This affords protection against hypoglycemia and thereby fulfils one of the most desirable features of any current or potentially new antidiabetic drug [41].

The present results demonstrate for the first time that Tyr^1-glucitol GIP displays profound resistance to serum and DPP IV degradation. Using ESI-MS the present study showed that native GIP was rapidly cleaved *in vitro* to a major 4748.4 Da degradation product, corresponding to GIP(3-42) which confirmed previous findings using matrix-assisted laser desorption ionization time-of-flight mass spectrometry [42]. Serum degradation was

completely inhibited by diprotin A (Ile-Pro-Ile), a specific competitive inhibitor of DPP IV, confirming this as the main enzyme for GIP inactivation *in vivo* [4,5]. In contrast, Tyr^1-glucitol GIP remained almost completely intact after incubation with serum or DPP IV for up to 12 hours. This indicates that glycation of GIP at the amino-terminal Tyr^1 residue masks the potential cleavage site from DPP IV and prevents removal of the Tyr^1-Ala^2 dipeptide from the N-terminus preventing the formation of GIP(3-42).

Consistent with *in vitro* protection against DPP IV, administration of Tyr^1-glucitol GIP significantly enhanced the antihyperglycemic activity and insulin-releasing action of the peptide when administered with glucose to rats. Native GIP enhanced insulin release and reduced the glycemic excursion as observed in many previous studies [12,40]. However, amino-terminal glycation of GIP increased the insulin-releasing and antihyperglycemic actions of the peptide by 62% and 38% respectively, as estimated from AUC measurements. Detailed kinetic analysis is difficult due to necessary limitation of sampling times, but the greater insulin concentrations following Tyr^1-glucitol GIP as opposed to GIP at 30 minutes post-injection is indicative of a longer half-life. The glycemic rise was modest in both peptide-treated groups and glucose concentrations following injection of Tyr^1-glucitol GIP were consistently lower than after GIP. Since the insulinotropic actions of GIP are glucose-dependent [30,40], it is likely that the relative insulin-releasing potency of Tyr^1-glucitol GIP is greatly underestimated in the present *in vivo* experiments.

In vitro studies in the laboratory of the present

inventors using glucose-responsive clonal B-cells showed that the insulin-releasing potency of Tyr^1-glucitol GIP was several order of magnitude greater than GIP and that it's effectiveness was more sensitive to change of glucose concentrations within the physiological range. Together with the present *in vivo* observations, this suggests that N-terminal glycation of GIP confers resistance to DPP IV degradation whilst enhancing receptor binding and insulin secretory effects on the B-cell. These attributes of Tyr^1-glucitol GIP are fully expressed *in vivo* where DPP IV resistance impedes degradation of the peptide to GIP(3-42), thereby prolonging the half-life and ehancing effective concentrations of the intact biologically active peptide. It is thus possible that glycated GIP is enhancing insulin secretion *in vivo* both by enhanced potency at the receptor as well as improving DPP IV resistance. Thus numerous studies have shown that GIP (3-42) and other N-terminally modified fragments, including GIP(4-42), and GIP (17-42) are either weakly effective or inactive in stimulating insulin release [4,43-45]. Furthermore, evidence exists that N-terminal deletions of GIP result in receptor antagonist properties in GIP receptor transfected Chinese hamster kidney cells [9], suggesting that inhibition of GIP catabolism would also reduce the possible feedback antagonism at the receptor level by the truncated GIP(3-42).

In addition to its insulinotopic actions, a number of other potentially important extrapancreatic actions of GIP may contribute to the enhanced antihyperglycemic activity and other beneficial metabolic effects of Tyr^1-glucitol GIP. These include the stimulation of glucose uptake in adipocytes, increased synthesis of fatty acids and activation of lipoprotein lipase in adipose

tissue [46-48]. GIP also promotes plasma triglyceride clearance in response to oral fat loading [49]. In liver, GIP has been shown to enhance insulin-dependent inhibition of glycogenolysis [50]. GIP also reduces both glucagon-stimulated lipolysis in adipose tissue as well as hepatic glucose production [51]. Finally, recent findings indicate that GIP has a potent effect on glucose uptake and metabolism in mouse isolated diaphragm muscle [52]. This latter action may be shared with tGLP-1 [53,54] and both peptides have additional benfits of stimulating somatostatin secretion and slowing down gastric emptying and nutrient absorption [1,55].

In conclusion, this study has demonstrated for the first time that the glycation of GIP at the amino-terminal Tyr^1 residue limits GIP catabolism through impairment of the proteolytic actions of serum petidases and thus prolongs its half-life *in vivo*. This effect is accompanied by enhanced antihyperglycemic activity and raised insulin concentrations *in vivo*, suggesting that such DPP IV resistant analogues should be explored alongside tGLP-1 as potentially useful therapeutic agents for NIDDM. Tyr^1-glucitol GIP appears to be particularly interesting in this regard since such amino-terminal modification of GIP enhances rather than impairs glucose-dependent insulinotropic potency as was observed recently for tGLP-1[28].

Detailed Figure Legends

Figure 1 illustrates degradation of GIP and Tyr^1-glucitol GIP by DPP IV. Representative HPLC profiles obtained after incubation of GIP (left panels) or Tyr^1-glucitol GIP exposed to DPP IV were separated on a

Vydac C-18 column using linear gradients of 0% to 31.5% acetonitrile over 15 minutes, to 38.5% over 30 minutes and from 38.5% to 70% acetonitrile over 5 minutes. Left hand panels show HPLC profiles of intact GIP (retention time 21.93 min) and GIP(3-42) (retention time 21.61 min), Right hand panels show HPLC profiles obtained for Tyr^1-glucitol GIP (retention time 21.75 min). HPLC peaks corresponding to intact GIP, GIP(3-42) and Tyr^1-glucitol GIP are indicated.

Figure 2 illustrates degradation of GIP and Tyr^1-glucitol GIP by human plasma. Representative HPLC profiles obtained after incubation of GIP (left panels) and Tyr^1-glucitol GIP (right panels) with human plasma for 0 and 4 hours and for 4 hours in the presence of 5mU of diprotin A(DPA). GIP and Tyr^1-glucitol GIP incubations were separated with a Vydac C-18 column using linear gradients 0% to 31.5% acetonitrile over 15 minutes, to 38.5% over 30 minutes and from 38.5% to 70% acetonitrile over 5 minutes. Peaks corresponding with intact GIP, GIP(3-42) and Tyr^1-glucitol GIP are indicated. A major peak corresponding to the specific DPP IV inhibitor tripeptide DPA appears in the bottom panels with retention time 16.29 min.

Figure 3 illustrates electrospray ionization mass spectrometry of GIP, Tyr^1-glucitol GIP and the major degradation fragment GIP(3-42). Samples containing (A)GIP, (B)Tyr^1-glucitol GIP and (C) the major degradation fragment of GIP (GIP(3-42)) isolated from plasma incubations (Figure 2). Peptides were dissolved (approximately 400 pmol) in 100μl of water and applied to the LC/MS equipped with a microbore C-18 HPLC column. Samples (30μl direct loop injection) were applied at a flow rate of 0.2ml/min, under isocratic conditions 35% acetonitrile/water. Mass spectra were

recorded using a quadripole ion trap mass analyzer. Spectra were collected using full ion scan mode over the mass-to-charge (m/z) range 150-2000. The molecular masses (M_r) of GIP and related structures were determined from ESI-MS profiles using prominent multiple charged ions and the following equation $M_r=iM_i-iM_h$ (see Research Design and Methods Section).

Figure 4 - effects of GIP and glycated GIP on plasma glucose homeostasis. **(A)** Plasma glucose concentrations after i.p. glucose alone (18mmol/kg) (control group), or glucose in combination with either GIP of Tyr^1-glucitol GIP (10nmol/kg). The time of injection is indicated by the arrow (0 min). **(B)** Plasma glucose AUC calues for 0-60 min post injection. Values are mean ± SEM for six rats. $^{**}P<0.01$, $^{***}P<0.001$ compared with GIP and Tyr^1-glucitol GIP; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ compared with non-glucated GIP.

Figure 5 - effects of GIP on plasma insulin responses (A). Plasma insulin concentrates after i.p. glucose along (18 mmol/kg) (control group), or glucose in combination with either with GIP or glycated GIP (10nmol/kg). The time of injection is indicated by the arrow. **(B)** Plasma insulin AUC values were calculated for each of the 3 groups up to 90 minutes post injection. The time of injection is indicated by the arrow (0 min). Plasma insulin AUC values for 0-60 min post injection. Values are mean ± SEM for six rats. $^{*}P<0.05$, $^{**}P<0.001$ compared with GIP and Tyr^1-glucitol GIP; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ compared with non-glycated GIP.

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Fig 1

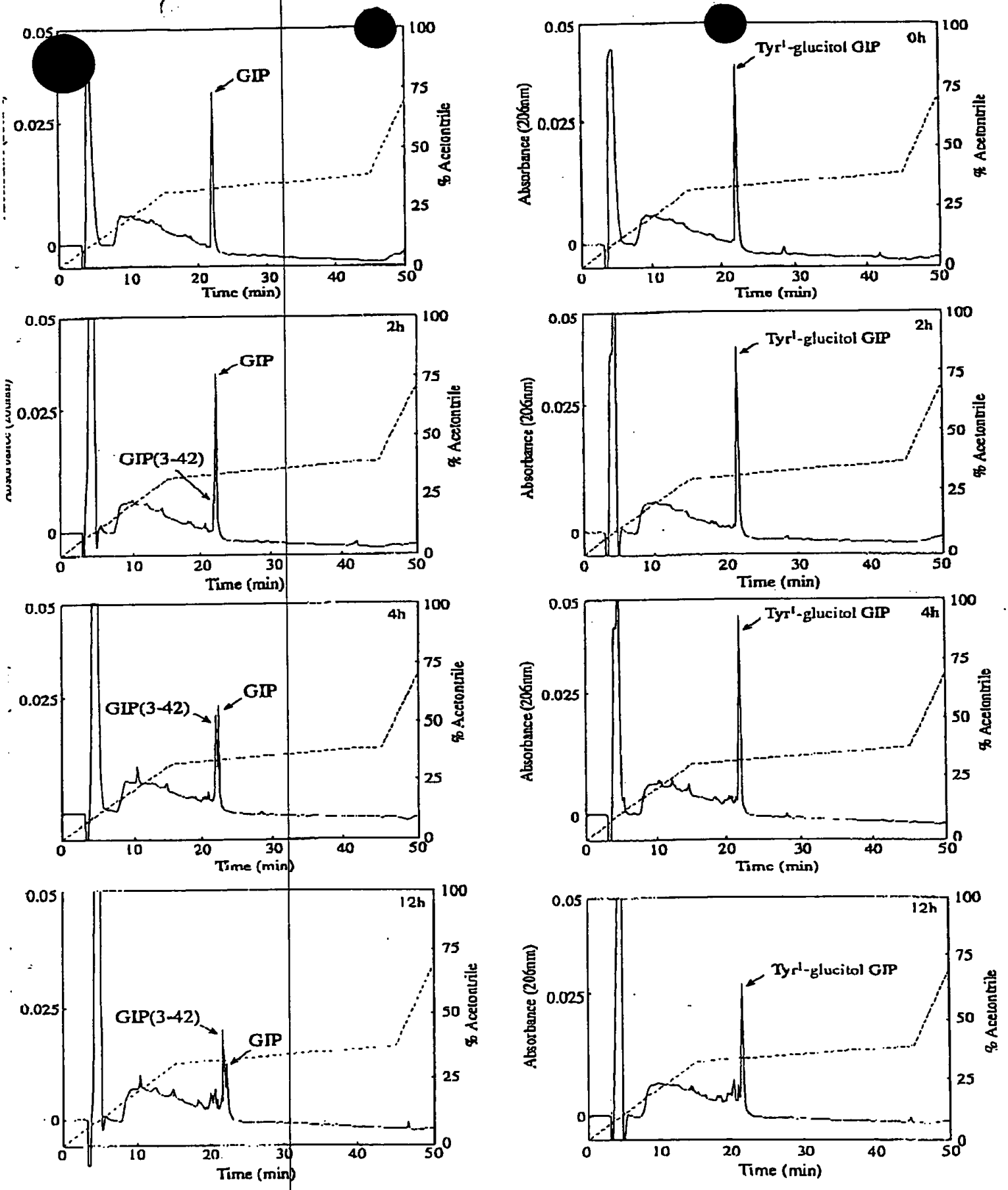

GIP
Tyr¹-glucitol GIP
0h
2h
4h
12h
GIP(3-42)
Absorbance (206nm)
% Acetonitrile
Time (min)
0.05
0.025
0
100
75
50
25
0
10
20
30
40
50

Fig 2

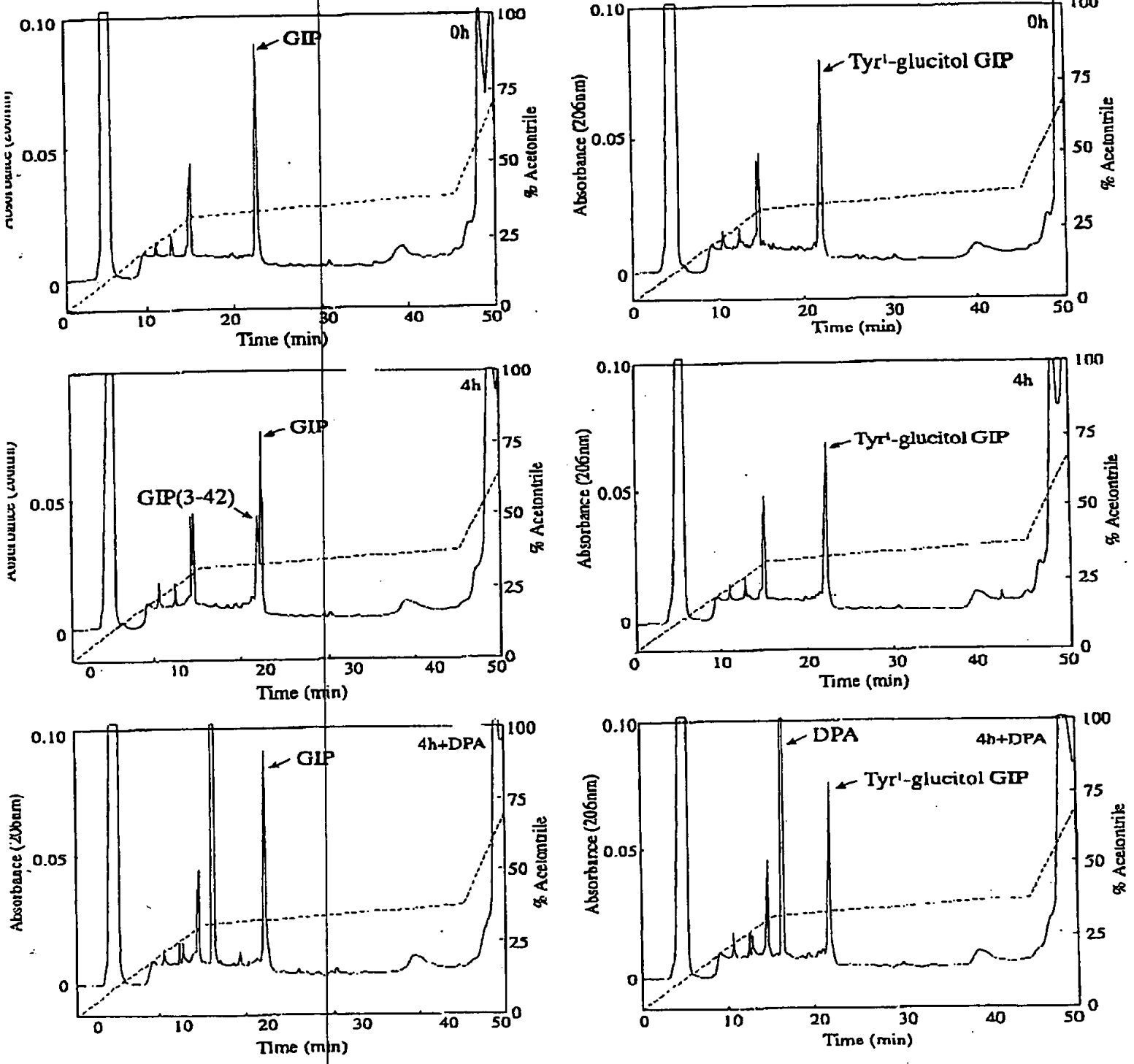

GIP
0h
Tyr¹-glucitol GIP
4h
GIP(3-42)
4h+DPA
DPA
Absorbance (206nm)
% Acetonitrile
Time (min)

Fig 3

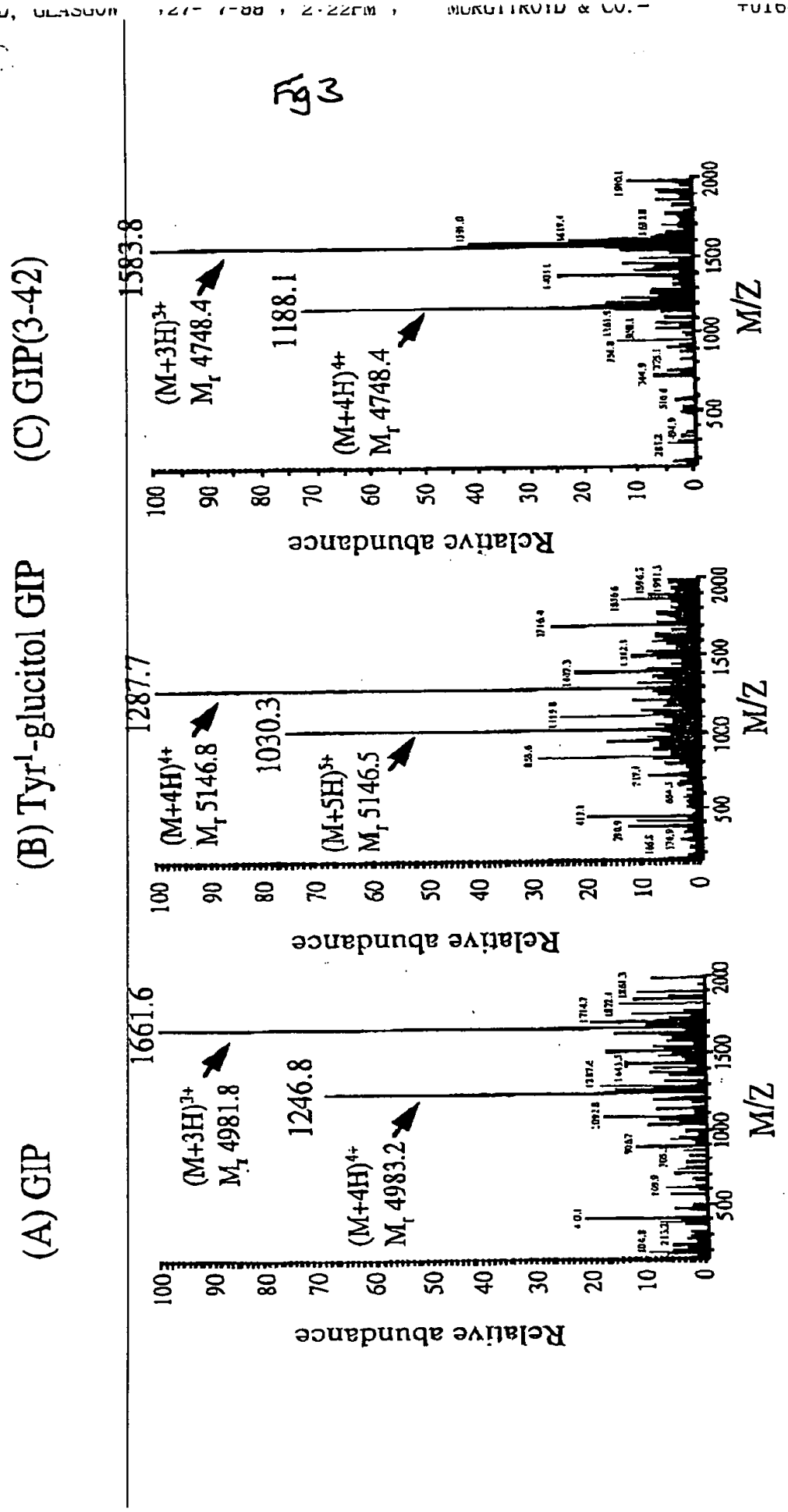

(A) GIP
(B) Tyr¹-glucitol GIP
(C) GIP(3-42)
Relative abundance
M/Z
1661.6
1246.8
(M+3H)³⁺ Mr 4981.8
(M+4H)⁴⁺ Mr 4983.2
1287.7
1030.3
(M+4H)⁴⁺ Mr 5146.8
(M+5H)⁵⁺ Mr 5146.5
1583.8
1188.1
(M+3H)³⁺ Mr 4748.4
(M+4H)⁴⁺ Mr 4748.4

Fig 4

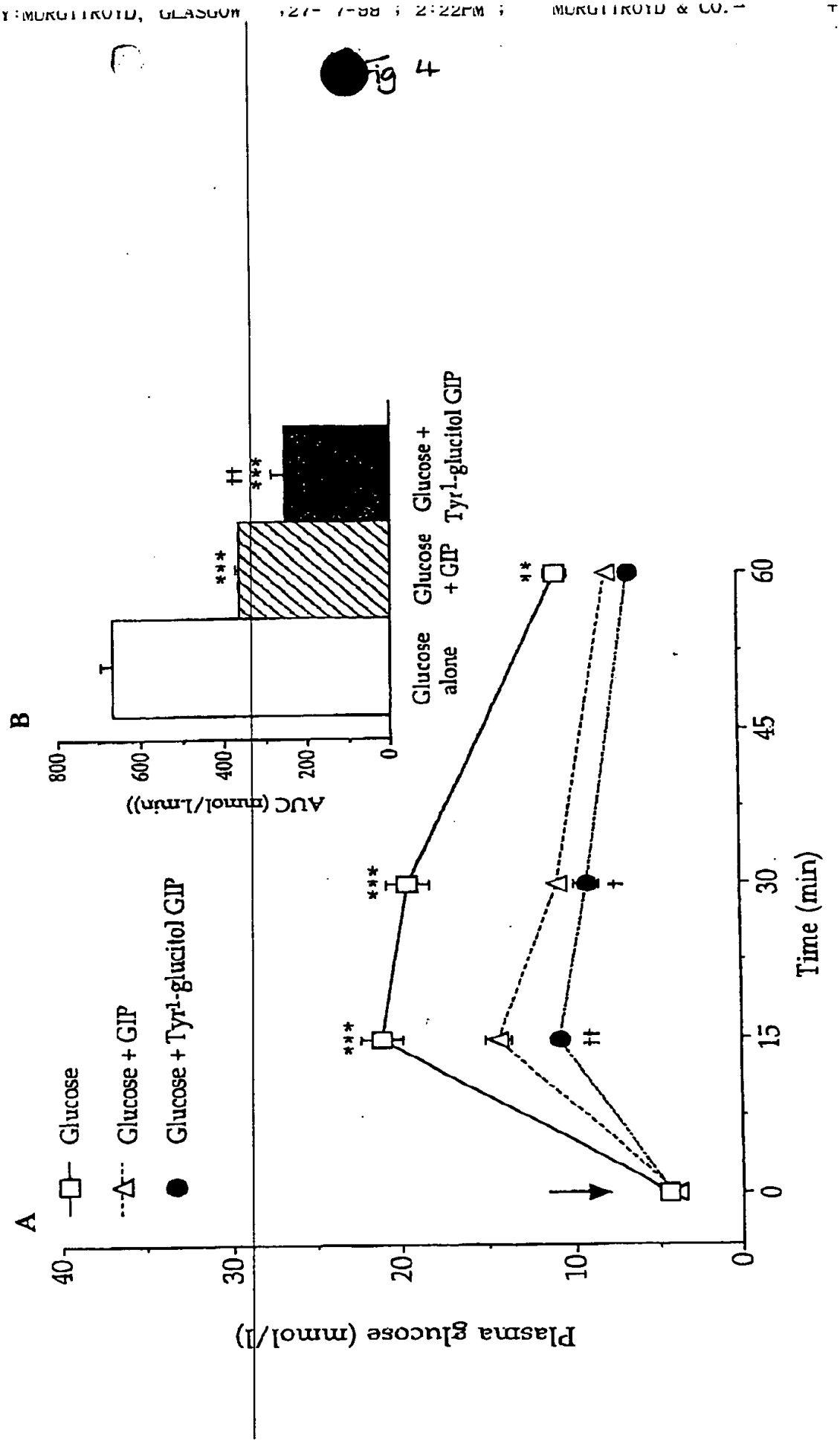
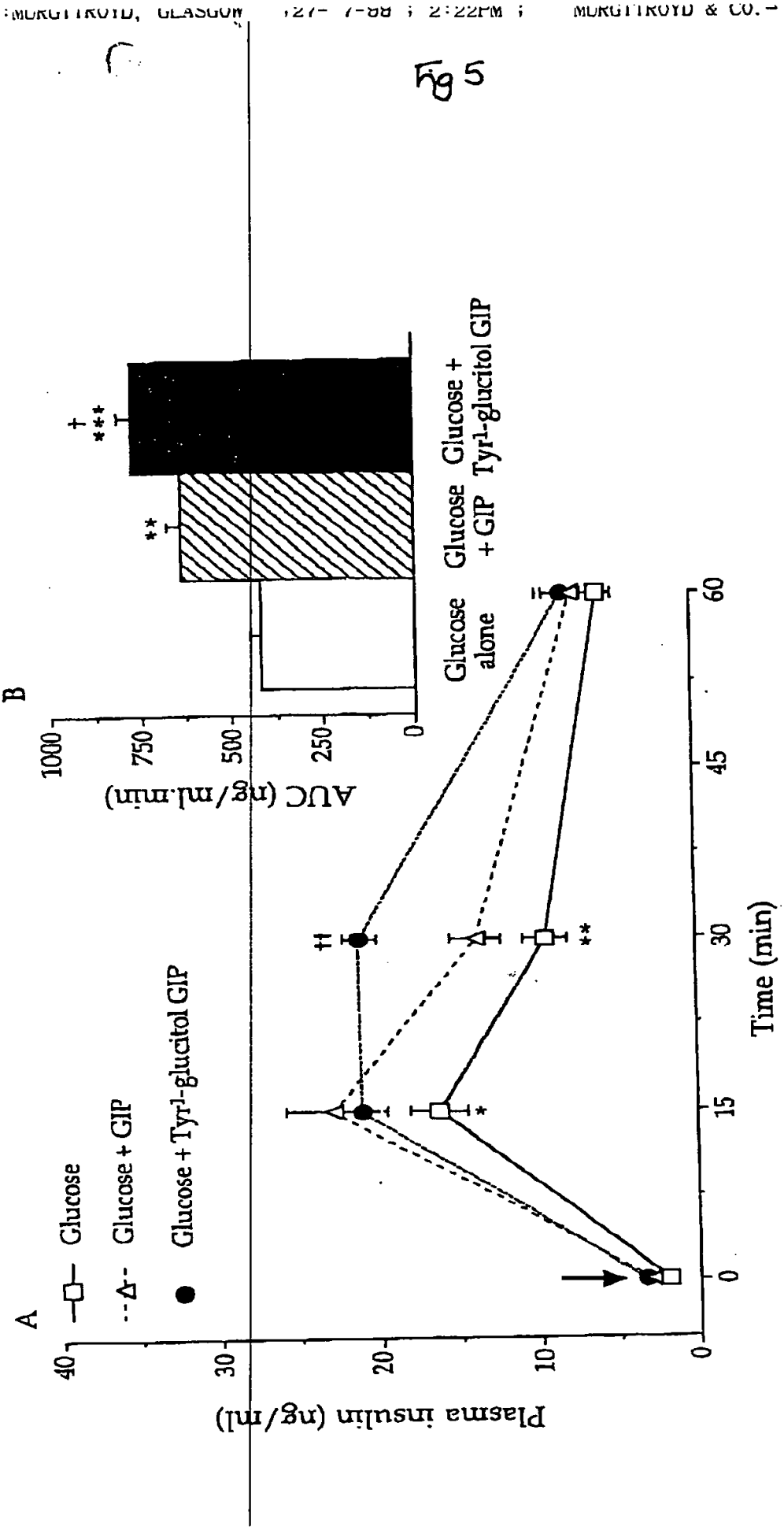

A
B
Glucose
Glucose + GIP
Glucose + Tyr1-glucitol GIP
Plasma glucose (mmol/l)
Time (min)
AUC (mmol/l.min))
Glucose alone
Glucose + GIP
Glucose + Tyr1-glucitol GIP

Fig 5


A
Glucose
Glucose + GIP
Glucose + Tyr1-glucitol GIP
Plasma insulin (ng/ml)
Time (min)
Glucose alone
Glucose + GIP
Glucose + Tyr1-glucitol GIP
B
AUC (ng/ml.min)

PCT GB 00 01089

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17/4/2000.